UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

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*Ex parte* BORGE KRINGELUM,
MAIBRITT KRINGEL,
and
KNUD STRIIB NIELSEN,
Appellants

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Appeal 2008-3378
Application 09/813,292[1]
Technology Center 1600

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Decided: August 7, 2008

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Before CAROL A. SPIEGEL, MARK NAGUMO, and LORA M. GREEN, *Administrative Patent Judges.*

SPIEGEL, *Administrative Patent Judge.*

DECISION ON APPEAL

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[1] Application 09/813,292 (the disclosure of which is cited herein as "Spec."), "Method For Supply of Starter Cultures Having A Consistent Quality," filed 21 March 2001, claims benefit under 35 U.S.C. § 119 of provisional application 60/191,307, filed 21 March 2000. The real party in interest is said to be Chr. Hansen A/S, Denmark (Appeal Brief, filed 6 February 2007 ("App. Br."), 1).

I. Statement of the Case

This is an appeal under 35 U.S.C. § 134 from a final rejection of all pending claims, claims 1-31. We have jurisdiction under 35 U.S.C. § 6(b). We AFFIRM.

The subject matter on appeal is directed to a method of using subsets of a concentrated stock inoculum material for direct inoculation of a culture medium to obtain a starter culture useful in the fermentation industry, e.g., for production of cheese. Claims 1 and 29 are illustrative and read (App. Br. 21 and 24, subparagraphing added):

> 1. A method of supplying starter cultures of consistent quality at different propagation factories or plants, comprising the steps of
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> (i) providing inoculum material comprising starter culture organism cells,
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> (ii) allowing the starter culture cells to propagate for a period of time sufficient to produce a desired amount of said starter culture organism cells, and
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> (iii) harvesting the propagated cells to obtain a starter culture,
>
> wherein step (i) comprises:
>
> (a) concentrating said inoculum material of step (i) to obtain a concentrated stock inoculum material;
>
> (b) dividing said concentrated stock inoculum material into subsets thereof and providing a subset to a different propagation factory or plant, each of said subsets having a quality sufficient to inoculate a cultivation

medium at different propagation factories or plants, and

(c) inoculating said cultivation medium at the different propagation factory or plant with the subset of the stock inoculum material by direct, one step inoculation to produce said starter culture,

wherein said stock inoculum material is subjected to a quality test before use and is stored for at least 24 hours prior to said inoculating of the cultivation medium,

such that, when steps (ii) and (iii) are repeated with another subset of the stock inoculum material at a different propagation factory or plant, the supply of starter cultures has a consistent quality.

29. The method of claim 1, wherein the stock inoculum material or a subset thereof is subjected to a quality test selected from the group consisting of Test for contamination, Count of total viable cells, Determination of colony morphology, Determination of purity, Determination of metabolic activity, Phage test, API test, Resistance to bacteriophages, Determination of the content of *Listeria* species and salmonella species, DNA fingerprint, and Fermentation test.

The Examiner relies on the following references[2] of record:

| | | |
|---|---|---|
| Christensen | 3,483,087 | Dec. 1969 |
| Rimler | 3,980,523 | Sep. 1976 |
| Czulak | 4,476,143 | Oct. 1984 |
| Kosikowski | 5,098,721 | Mar. 1992 |
| Matsumiya | 5,225,346 | Jul. 1993 |
| Lizak | 5,952,020 | Sep. 1999 |
| Vandenbergh | 6,068,774 | May 2000 |
| Sing | 6,146,667 | Nov. 2000 |

The Examiner has rejected

(i) claims 1-7, 11, 17-22, and 24-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski and Christensen (FR[3] 3-6; Ans.[4] 3-8);

(ii) claims 1-7, 11, 17-22, 24-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, and Czulak (FR 6-10; Ans. 8-11);

(iii) claims 1-11, 17-22, 24-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, and Lizak (FR 10-14; Ans. 12-15);

(iv) claims 1-7, 11-22, 24-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, Vandenbergh, and Matsumiya (FR 14-18; Ans. 16-20, 30);

(v) claims 1-7, 11, 17-22, 24-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, Czulak, and Lizak (FR 18-22; Ans. 20-24, 30); and,

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[2] No references to *et al.* are made in this Decision.
[3] Final Office Action mailed 7 July 2006 ("FR").
[4] Examiner's Answer mailed 27 June 2007 ("Ans.").

(vi) claims 1-7, 11, 17-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, Rimler, and Lizak (FR 22-26; Ans. 24-28, 30).

In the Answer, the Examiner clarified that Christensen was relied on in each of the rejections whether or not the rejection recited Christensen in the heading (Ans. 30).

Appellants have separately argued dependent claim 29. Therefore, the patentability of dependent claims 2-28 and 30-31 stand or fall with the patentability of independent claim 1 from which they depend. We decide this appeal on the basis of claims 1 and 29. 37 C.F.R. § 41.37(c)(1)(vii).

In summary, Appellants argue Sing and the secondary references fail to suggest supplying starter cultures of consistent quality *at different propagation factories or plants* as recited in claim 1 (App. Br. 8, 16-19). As to claim 29, Appellants argue lack of motivation to use Christensen's quality tests "which are entirely different from the quality tests of the claimed method in the aspects of type, standard and purpose" (App. Br. 15-16).

II. Opinion

Findings of fact set forth in this opinion are supported by a preponderance of the evidence of record.

A. Appellants' invention

According to the 292 specification, microbial cultures are used extensively for fermentations in the manufacturing of food, feed, pharmaceutical products, enzymes, and metabolites (Spec. 1:15-17). Most fermentations are said to be based on the use of dried, frozen or freeze-dried microbial inoculation media (Spec. 1:20-21). Inoculation material is said to

be produced in small ampules for distribution to fermentation plants (Spec. 1:21-22).

The 292 specification describes a conventional preparation of a working or "starter" culture as progressing in a series of steps from inoculating a culture medium with a primary inoculum material to obtain a mother culture, through transferring the resulting culture through a series of 2-4 intermediate cultures prepared in increasingly larger volumes, until a sufficient amount of inoculum material is obtained to use as a working or starter culture (Spec. 1:30-35).

In contrast, the 292 specification describes a process wherein the primary inoculum material is concentrated and divided into storable subsets wherein each one of the subsets is used to provide enough inoculum material directly to a fermenter with culture medium to provide a starter culture, i.e., a "one-step, direct inoculation" process (Spec. 4:24-5:7; 6:10-16). Since the subsets originate from the same concentrated stock inoculum, the starting material for different starter cultures is said to be substantially the same and of a high and consistent quality (Spec. 4:33-5:2). Thus, according to the 292 specification, "it is possible to reduce the variation of the product quality both from batch to batch, but also between factories and plants within a given company" (Spec. 17:30-31). The 292 specification defines a "concentrate" as "a suspension of cells or medium comprising the cells . . . having a content of viable cells (colony forming units, CFUs) which is at least $10^8$ CFU per g" (Spec. 8:12-15).

B. The prior art

Sing discloses a method of preparing starter cultures for inoculating milk to manufacture cheese and other cultured dairy products (Sing 1:9-11, 46-

48). "One preferred method generally comprises: (a) introducing an inoculum having at least about $10^{11}$ CFU/g to a growth medium to produce an inoculated medium having at least about $10^{7}$ CFU/g; and (b) growing the inoculated medium to produce a starter culture having at least about $10^{9}$ CFU/g" (Sing 1:48-52). The starter culture is then added to milk to produce a cultured dairy product (Sing 1:52-54). According to Sing, use of ultra-high culture inoculum (at least about $10^{11}$ CFU/g or approximately 60-100 times greater than prior art cultures) reduces preparation time, liberates vat space in plants, and/or allows for preparation of other products or allows for emergency preparation within the same day of production (Sing 2:48-51; 3:51-62)

Kosikowski discloses (Kosikowski 1:18-37):

> there are many distinct types of starter cultures for milk fermentations . . . . A mother culture is a small volume of inoculated growth medium, . . . which is periodically transferred, usually daily, into a plurality of growth medium containers with the best resulting cultures selected for making a larger volume of starter, . . . . The mother starter is synonymous with certified seed. Bulk starter, . . . can be made from the mother starter and is used for inoculation of the milk product as a part of the production fermentation step. The mother or bulk starter can be stored in . . . a dry state or powder, . . . [or] frozen and the frozen product . . . can be in the concentrated form.

Christensen discloses that the starter culture "has greater influence on the quality of the cheese than any other factor. . . . Consequently, extreme care must be taken not only in producing the cheese starter cultures but also in maintaining them in good condition until ready for use in the

cheesemaking process" (Christensen 1:44-53). Christensen monitors the uniformity and stability of cultures after each transfer using at least 3 of the following tests -- a standard acid test, an activity test run at 89°F and/or at 103°F, a gas test after the first and fourth transfer, and a standard plate count test (Christensen 5:73-7:3).

A discussion of Rimler, Czulak, Matsumiya, Lizak, and Vandenbergh is not necessary to our decision.

C. Examiner's findings and conclusions

The Examiner found Sing teaches a method of making starter for inoculating milk to make dairy products (FR 4, 8, 11, 15, 19, 23; Ans. 6, 9, 13, 17, 21, 25).

The Examiner found Sing does not teach a method wherein the primary inoculum material is first concentrated and divided into subsets (FR 5, 8, 12, 16, 20, 24; Ans. 6, 10, 13, 18, 22, 26). The Examiner concluded "it would have been obvious . . . to concentrate and/or divide the inoculum into subsets as a matter of routine practice and procedure" (FR 5, 8, 12, 16, 20, 24; Ans. 6, 10, 13-14, 18, 22, 26) because Kosikowski teaches concentrating mother culture for storage and transferring, i.e., dividing, mother cultures into multiple growth media (FR 5, 8, 12, 16, 20, 24; Ans. 6, 10, 14, 18, 22, 26).

The Examiner found that neither Sing nor any of the secondary references teaches a method wherein the subsets are provided to different factories and/or plants (FR 5, 9, 13, 17, 21, 24; Ans. 7, 11, 14, 19, 23, 26). The Examiner concluded "the location of where the actual steps take place do not patentably distinguish the method from the prior art, since practicing

the methods at different locations would not materially change the culture method" (FR 5-6, 9, 13, 17, 21, 24-25; Ans. 7, 11, 14, 19, 23, 26-27).

The Examiner found that Sing does not teach subjecting the inoculum to quality tests before use or storing the inoculum for 24-48 hours before use (FR 5, 8, 12, 16, 20, 24; Ans. 7, 10, 14, 18, 22, 26). The Examiner concluded "it would have been obvious . . . to do so because quality tests where [sic] routinely employed in the art" (FR 5, 8, 12, 16, 20, 24; Ans. 7, 10, 14, 18, 22, 26) as shown by the acid, activity, gas, and CFU plate counting tests disclosed by Christensen (FR 5, 8-9, 12, 16, 20, 24; Ans. 7, 10, 14, 18, 22, 26).

D. Appellants' arguments

Appellants argue the subject matter of claim 1 "is not the biochemistry occurring in a single vat but is a *method of supplying starter cultures of consistent quality at different propagation factories or plants*" (App. Br. 9, original emphasis). According to Appellants (App. Br. 11)

> an exemplary implementation of the conventional approach to making starter cultures would have entailed, first, preparing one liter of culture, by propagating bacterial cells until the growth of the cells in the medium ceased. That culture would have been transferred to another fermenter, some 100 times larger in volume (100 L), and the cells again propagated until the growth stopped. Thereafter, the resulting 100 liters of culture would have been transferred to the final fermenter, with a volume of 10,000 liters in which the cells would have propagated until growth stopped. The cells then would have been harvested and disseminated as starter cultures for the dairy industry.

According to Appellants, performing these multiple growth steps at different factories or plants or at different times in the same factory/plant presents multiple, unique opportunities for contamination, mutation, and variation among the different starter cultures obtained (App. Br. 11).

Appellants rely on a Declaration of Borge Kringelum, dated February 17, 2004, ("Declaration") to support their position. Mr. Kringelum testified he is one of the inventors and a dairy engineer employed by the real party in interest (Declaration ¶ 1). Mr. Kringelum stated he compared 457 batches of conventionally produced commercial starter culture with 115 batches of starter culture produced by the claimed method with regard to the percentage of batches "approved" (Declaration ¶ 6). According to Mr. Kringelum, "product approval increased by 5.25% as a consequence of producing the batches according to the claimed invention" (Declaration ¶ 7). Further according to Mr. Kringelum, "[u]nder the probable assumption that the increased approval rate of 5.25% resulting from a production period lasting two years is representative of our production factories world-wide, a total global cost saving of 1.5 million US$ per year is predictable" (Declaration ¶ 7).

As to claim 29, Appellants specifically argue the purpose of the quality tests in the claimed method is to ensure a high quality starter culture free of contamination, whereas the purpose of the quality tests in Christensen is to ascertain the cheese-making qualities of the culture (App. Br. 15). Thus, Appellants contend there is no motivation to use the quality tests of Christensen in the claimed method (App. Br. 15-16).

E. Legal principles

A claimed invention is not patentable if it would have been obvious to a person having ordinary skill in the art. 35 U.S.C. § 103(a); *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct., 1727 (2007); *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1 (1966). Facts relevant to a determination of obviousness include (1) the scope and content of the prior art, (2) any differences between the claimed invention and the prior art, (3) the level of skill in the art, and (4) any relevant objective evidence of obviousness or non-obviousness. *KSR*, 127 S.Ct. at 1734; *Graham*, 383 U.S. at 17-18.

"[I]n proceedings before the PTO, claims in an application are to be given their broadest reasonable interpretation consistent with the specification." *In re Sneed*, 710 F.2d 1544, 1548 (Fed. Cir. 1983). The scope of claim language is not limited to the preferred embodiments described in its supporting specification.

F. Claim 1 analysis

Here, Appellants contend "one cannot reasonably equate a 'propagation factory' with a 'growth medium'" (Reply Br.[5] 6). However, the 292 specification does not contain an explicit definition of a "propagation factory" or a "propagation plant." Although the 292 specification describes embodiments wherein the stock inoculum material provided in claim 1, step (i), is in quantities sufficient to inoculate between at least about 20 kg of cultivation medium and at least 180,000 kg of cultivation medium (Spec. 5:27-6:2), claim 1 does not recite such limitations.

[5] Reply Brief filed 27 August 2007 ("Reply Br.").

Rather, according to the 292 specification (Spec. 7:16-21),

> the term "propagation" is used interchangeably with the terms "cultivation" and "fermentation" and refers to the broadest sense of these terms with respect to processes whereby biomasses of production strains are obtained. The term "production strain" refers, in the present context, to cells of any microbial species that can be used in industrial productions of starter cultures including species of bacteria including lactic acid bacteria, fungi, and yeast.

Further according to the 292 specification, "it is generally preferred to add an amount of the inoculum material to the cultivation medium that provides a number of CFUs which is at least $10^5$ CFUs per g of cultivation medium immediately after inoculation. . . ." (Spec. 9:11-14). As noted above, Sing discloses a method comprising introducing an inoculum having at least about $10^{11}$ CFU/g to a growth medium to produce an inoculated medium having at least about $10^7$ CFU/g (Sing 1:48-52). Thus, we find ample support in the record for the Examiner's conclusion that "[e]ach growth medium could reasonably be interpreted as its own propagation factory of the starter culture as each medium is in a separate location" (Ans. 29). Furthermore, as stated by the Examiner (Supp. Ans. 5),

> Kosikowski . . . teaches common practices wherein mother cultures (starter cultures) are transferred (or inoculated) into multiple growth media. Each growth medium could reasonably be interpreted as its own propagation factory of the starter culture as each medium is in a separate location.

We also find the record supports the Examiner's finding that Sing specifically teaches a direct, one step inoculation as encompassed by claim

1, step (i) (Supp. Ans.[6] 3). Thus, arguments based on a narrow interpretation of "different propagation factories or plants" as recited in claim 1 are unpersuasive of Examiner error in her *prima facie* conclusion of obviousness. The Kringelum Declaration does not convince us otherwise.

Appellants argue the declaration shows a significantly higher approval percentage across a broad spectrum of cultures is obtained with the claimed methodology (Surreply 5-6). However, only data limited to gram-positive lactic acid bacteria ("LAB") is presented in the declaration.[7] The method of claim 1 encompasses any starter culture organism cells, including gram-positive and gram-negative bacteria, fungi, and yeast (see dependent claim 17). Moreover, six of the seven LAB tested belong to the same *Clostridium* branch of gram-positive bacteria (*B. bifidum* belongs to the *Actinomyces* branch of gram-positive bacteria). Thus, as noted by the Examiner (Supp. Ans. 4), the data in the declaration is not commensurate in scope with the claims. Further, although Mr. Kringelum characterizes the data based on "the method of the present invention," he did not testify the data was based on the claimed method.

Appellants argue the data in the declaration are comparable (Surreply 6-7). In particular, Appellants argue "[o]ne skilled in the art would have appreciated that statistical method allows one to compare the average of two observables, A and B, even if one measures observable A fewer times than observable B" (Surreply 6). However, Mr. Kringelum did not explain what testing procedure was used to test for the number of cells, metabolic activity,

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[6] Supplemental Examiner's Answer mailed 25 October 2007 ("Supp. Ans.").
[7] See e.g., Hammes et al., "The potential of lactic acid bacteria for the production of safe and wholesome food," *Z. Lebensm Unters Forsch*, 198: 193-201, at 195 (1994): (copy enclosed).

or presence of bacterial contamination, whether the same tests were run on all batches tested, whether the same positive and/or negative controls were used, whether the tests were recognized standard protocols in the art, etc. Thus, the declaration provided no statistical basis for interpreting the raw data presented in the declaration or ascertaining what defines a "significantly higher" acceptance rate. For example, the data might have been subject to a standard error of 1% or 5% or 10% and/or standard error might differ from microorganism to microorganism or from genus to genus, depending upon characteristics of the genus, such as percentage of G+C in their DNA or the ability to tolerate oxygen, or the type of culture medium used. Therefore, as noted by the Examiner (Supp. Ans. 4), the raw data presented in Table 1 of the declaration is unclear.

According to the Examiner, "the data [in the declaration] does not appear to evidence that a culture with consistently high quality is an unexpected result, especially since the prior art suggests a consistent quality is achieved" (Supp. Ans. 4, bracketed text added). The Examiner relies on Sing to support her position (Ans. 7). Sing discloses a method generally comprising introducing an inoculum having at least about $10^{11}$ CFU/g to a growth medium to produce an inoculated medium having at least about $10^{7}$ CFU/g and growing the inoculated medium to produce a starter culture having at least about $10^{9}$ CFU/g (Sing 1:48-52). According to Sing, use of ultra-high culture inoculum reduces preparation time and circumvents the need for phage inhibition (Sing 3:51-62). One of ordinary skill in the art would have reasonably expected the method of Sing to produce a consistently high quality starter culture since reducing the number of culture steps, transfers, and times would concomitantly reduce errors that may occur

in the deleted steps over time. Furthermore, it is not clear that Mr. Kringelbum compared the claimed method to what appears to be the closest prior art, i.e., the Sing method. Therefore, contrary to Appellants' argument (Surreply 7-8), the Examiner does not appear to be taking inconsistent positions.

For the foregoing reasons, the Kringelbum Declaration is insufficient to overcome the Examiner's *prima facie* conclusion of obviousness.

Based on the foregoing, we affirm the rejections of (i) claims 1-7, 11, 17-22, 24-28, 30, and 31 under § 103(a) over Sing in view of Kosikowski and Christensen; (ii) claims 1-7, 11, 17-22, 24-27, 30, and 31 under § 103(a) over Sing in view of Kosikowski, Christensen, and Czulak; (iii) claims 1-11, 17-22, 24-27, 30, and 31 under § 103(a) over Sing in view of Kosikowski, Christensen, and Lizak; (iv) claims 1-7, 11-22, 24-27, 30, and 31 under § 103(a) over Sing in view of Kosikowski, Christensen, Vandenbergh, and Matsumiya; (v) claims 1-7, 11, 17-22, 24-27, 30, and 31 under § 103(a) over Sing in view of Kosikowski, Christensen, Czulak, and Lizak; and, (vi) claims 1-7, 11, 17-27, 30, and 31 under § 103(a) over Sing in view of Kosikowski, Christensen, Rimler, and Lizak.

G. Claim 29 analysis

Claim 29 requires the stock inoculum or subset thereof used in the method of claim 1 to be selected from a Markush group of eleven tests, including a count of the total number of viable cells per g of culture or CFU/g.

Appellants do not challenge the Examiner's finding that Christensen also discloses CFU plate counting tests (Ans. 7, 10, 14, 18, 22, 26, 30). Instead, Appellants argue there is no motivation to use Christensen's quality

tests in the claimed method because Christensen's quality tests differ in type, standard, and purpose (App. Br. 15-16). Specifically, "[t]he claimed tests not only are for a different purpose but also encompass a much broader scope relative to the tests disclosed in the prior art" (Reply Br. 9).

Claim 29 does not require *all* of the recited tests to be performed, only one, e.g., the CFU plate counting test disclosed by Christensen. According to the Examiner, Christensen teaches its disclosed quality tests "ensure quality of the starter culture itself" (Ans. 30). Sing, for example, uses ultra-high culture inocula having at least about $10^{11}$ CFU/g and starter cultures having at least about $10^{9}$ CFU/g. Their combined teachings suggest using the CFU plate counting test of Christensen to ensure the quality of the starter culture of Sing. To hold otherwise presumes that a person of ordinary skill in the art is unknowledgeable and unskilled. This is error. *In re Sovish*, 769 F.2d 738, 743 (Fed. Cir. 1985). "A person of ordinary skill in the art is also a person of ordinary creativity, not an automaton." *KSR*, 127 S.Ct. at 1742.

It is not necessary to our decision to discuss additional quality tests.

Based on the foregoing, we affirm the rejections of claim 29 under § 103(a) over (i) Sing in view of Kosikowski and Christensen; (ii) Sing in view of Kosikowski, Christensen, and Czulak; (iii) Sing in view of Kosikowski, Christensen, and Lizak; (iv) Sing in view of Kosikowski, Christensen, Vandenbergh, and Matsumiya; (v) Sing in view of Kosikowski, Christensen, Czulak, and Lizak; and, (vi) over Sing in view of Kosikowski, Christensen, Rimler, and Lizak.

III. Order

Upon consideration of the record, and for the reasons given, it is

ORDERED that the decision of the Examiner rejecting claims 1-7, 11, 17-22, and 24-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski and Christensen is AFFIRMED;

FURTHER ORDERED that the decision of the Examiner rejecting claims 1-7, 11, 17-22, 24-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, and Czulak is AFFIRMED;

FURTHER ORDERED that the decision of the Examiner rejecting claims 1-11, 17-22, 24-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, and Lizak is AFFIRMED;

FURTHER ORDERED that the decision of the Examiner rejecting claims 1-7, 11-22, 24-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, Vandenbergh, and Matsumiya is AFFIRMED;

FURTHER ORDERED that the decision of the Examiner rejecting claims 1-7, 11, 17-22, 24-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, Czulak, and Lizak is AFFIRMED;

FURTHER ORDERED that the decision of the Examiner rejecting claims 1-7, 11, 17-27, and 29-31 as unpatentable under 35 U.S.C. § 103(a) over Sing in view of Kosikowski, Christensen, Rimler, and Lizak is AFFIRMED; and,

FURTHER ORDERED that no time period for taking any subsequent action in connection with this appeal may be extended under 37 C.F.R. § 1.136(a)(1)(iv).

AFFIRMED

rvb

Enc.: Hammes et al., "The potential of lactic acid bacteria for the production of safe and wholesome food," *Z. Lebensm Unters Forsch*, 198 (1994):193-201.

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| Notice of References Cited | Application/Control No. 09/813,292 | Applicant(s)/Patent Under Reexamination of a Patent Appeal No. 2007-3229 | |
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Z Lebensm Unters Forsch (1994) 198:193–201

Zeitschrift für
Lebensmittel-
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und -Forschung



*Review*

# The potential of lactic acid bacteria for the production of safe and wholesome food

**Walter P. Hammes, Petra S. Tichaczek**

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Received October 11, 1993

**Das Potential der Milchsäurebakterien bei der Produktion von hygienisch sicheren und gesundheitsfördernden Lebensmitteln**

**Zusammenfassung.** Milchsäurebakterien sind für die Herstellung fermentierter Lebensmittel von wesentlicher Bedeutung, und ihre Anwendung hat eine weit zurückreichende Tradition. Fermentierte Lebensmittel stellen ein Viertel unserer Nahrung und zeichnen sich u.a. dadurch aus, daß sie gegenüber der Rohware länger haltbar sind. Zusätzlich sollen diese Lebensmittel durch die Fermentation mit Milchsäurebakterien bestimmte gesundheitsfördernde Eigenschaften erwerben, die auf folgende Stoffwechselleistungen zurückgeführt werden:

1. Bildung von Milchsäure und in geringerem Umfang von Essigsäure und Ameisensäure. Diese Säuren bewirken eine Absenkung des pH-Wertes im Lebensmittel und damit wird das Wachstum unerwünschter Keime unterdrückt; bestimmte pathogene Mikroorganismen werden abgetötet und schädliche Pflanzeninhaltsstoffe werden inaktiviert.
2. Bildung antimikrobiell wirksamer Substanzen (z.B. Bakteriozine, $H_2O_2$, Fettsäuren).
3. Direkte Wirkung als Probiotika in Lebensmitteln.

Die förderlichen Effekte der Milchsäurebakterien können sich auch auf Bereiche der Tier- und Pflanzenzüchtung erstrecken. Die Konzepte für die Anwendung der Milchsäurebakterien im Lebens- und Futtermittelbereich schließen folgendes ein:

1. Selektion der best-adaptierten und reproduzierbar wirkenden Stämme.
2. Selektion der Stämme mit probiotischen Effekten.
3. Selektion von Stämmen, die im Lebensmittel gesundheitsfördernd wirken (durch Bildung von Vitaminen, essentiellen Aminosäuren bzw. Antitumoraktivität).
4. Selektion der Stämme, die als Schutzkulturen Verderbskeime und pathogene Mikroorganismen in Lebensmitteln hemmen.

Diese Stämme können den unterschiedlichen Lebensmitteln zugesetzt oder als Starterorganismen bei Lebensmittelfermentationen verwendet werden. Dabei kann es sich entweder um Mikroorganismen handeln, die aus Lebensmitteln isoliert oder durch genetische Veränderung erzeugt wurden. Die Methoden zur Änderung des genetischen Potentials dieser Organismen sind in jüngster Zeit entwickelt worden.

**Abstract.** By tradition lactic acid bacteria (LAB) are involved in the production of fermented foods. These constitute one quarter of our diet and are characterized by a safe history, certain beneficial health effects, and an extended shelf life when compared with raw materials. The various fermenting substrates are habitats for specific LAB that differ in their metabolic potential. The health effects exerted by LAB are the following:

1. Production of lactic acid and minor amounts of acetic and formic acid. These cause: a drop in pH and thereby growth inhibition of food spoiling or poisoning bacteria; killing of certain pathogens; detoxification by degradation of noxious compounds of plant origin (usually in combination with plant-derived enzymatic activities).
2. Production of antimicrobial compounds (e.g. bacteriocins, $H_2O_2$, fatty acids).
3. Probiotic effects as live organisms in food.

The wholesomeness of LAB can also be extended to fields outside human nutrition, as they may act as probiotics in animal production or as plant protectives in agriculture and thus contribute to healthy raw materials for food production. Modern concepts or perspectives of the application of LAB include the following:

1. Selection of the best adapted and safely performing LAB strains.
2. Selection of strains with probiotic effects.
3. Selection of strains with health-promoting effects (e.g. production of vitamins or essential amino acids, antitumour activity).

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4. Selection of strains with food protective activities (inhibiting spoilage or food pathogens).

These strains can be added to food or used as starters in food fermentations. They may be found as wild-type organisms or can be obtained by genetic engineering. Methods for modification of their genetic potential have been developed.

## Introduction

Most of the methods of food preservation date back to prehistoric times and have proved their safe and beneficial nature. The fermentation of food is included in these methods, and the fermented products belong to our most valuable foods since they are characterized by the following unique properties: they are ready to eat, rich in flavour, naturally preserved, hygienically safe, produced with low energy input and they have a high nutritive value. Among the micro-organisms involved in the fermentation process, lactic acid bacteria (LAB) are most important since they participate in the fermentation of a large variety of foods.

In Table 1 are listed foods fermented by LAB either alone or in combination with other groups of micro-organisms. The safe nature of the fermented foods implies that the LAB involved in their production share this beneficial property and, therefore, these organisms have received growing interest. Especially during the past decade LAB have been intensively studied with regard to their taxonomy, ecology, physiology, application, molecular biology and accessibility to gene technology. The purpose of this communication is to present an overview of the important practical aspects that may contribute to an understanding of the effects of LAB in obtaining safe and wholesome food.

Table 1. Foods of plant and animal origin fermented by lactic acid bacteria (LAB) either alone or in combination with other groups of micro-organisms [1–8]

| Substrate | Product | LAB involved* |
|---|---|---|
| *Plant origin* | | |
| Olives | Fermented olives | *Leuconostoc mesenteroides* |
| Cabbage | Sauerkraut | *Lactobacillus bavaricus* |
| Cucumber | Pickled cucumber | *L. brevis* |
| Tomatoes and minor important substrates, e.g., celery, eggplants, levant garlic, green beans | Fermented vegetables | *L. curvatus*<br>*L. plantarum*<br>*L. sake*<br>*Pediococcus pentosaceus* |
| Doughs or batters made from flour | Sourdough | *L. sanfrancisco*<br>*L. brevis*<br>*L. pontis sp. nov.* |
| | Kisra sourdough | *L. reuteri*<br>*L. fermentum*<br>*L. amylovorus* |
| Must or wine | Malolactic fermented wine | *Leuconostoc oenos* |
| Soy | Soy sauce | *Tetragenococcus halophilus* |
| *Animal origin* | | |
| Milk | Cultured buttermilk | *L. mesenteroides ssp. cremoris*<br>*Lactococcus lactis ssp. cremoris* |
| | Yoghurt | *L. lactis ssp. diacetylactis* |
| | Cheese | *Lactobacillus delbrueckii ssp. bulgaricus*<br>*Streptococcus thermophilus*<br>*Lactococcus lactis ssp. lactis*<br>*L. lactis ssp. diacetylactis*<br>*Leuconostoc mesenteroides ssp. cremoris*<br>*Lactobacillus helveticus*<br>*L. delbrueckii ssp. lactis*<br>*L. casei*<br>*L. plantarum* |
| Meat | Fermented sausages | *L. curvatus*<br>*L. plantarum*<br>*L. sake*<br>*P. acidilactici*<br>*P. pentosaceus* |
| Fish | Fish sauce | *T. halophilus* |

* In the course of the fermentation process these species occur at varying ratios

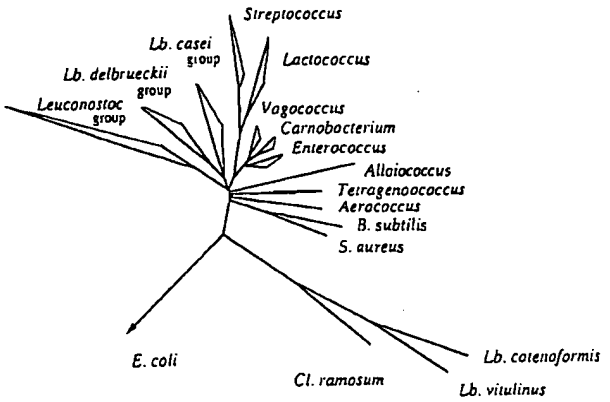


**Fig. 1.** Phylogenetic relationship of lactic acid bacteria (LAB) based on 16S rRNA analysis ([10], with permission): *Lb., Lactobacillus; B., Bacillus; S., Staphylococcus; E., Escherichia; Cl., Clostridium*

## Taxonomy of LAB

The taxonomy of LAB has recently been investigated very thoroughly [9,10]. In Fig. 1 the evolutionary relationship of the genera of LAB is depicted. It can be derived that several new genera have been added to the body of genera known from the time when Orla-Jensen [11] introduced the first grouping of LAB. Furthermore, the genera *Lactococcus, Leuconostoc, Lactobacillus* and *Pediococcus,* as the classical groups of organisms involved in food fermentation, are evolutionarily well-defined. They do not include any species with the potential for pathogenicity or toxicity. The genera are Gram-positive and are located in the so called *Clostridium* branch of evolution [12], which is characterized by a low (< 55%) percentage of G + C in their DNA. One group, the bifidobacteria, belongs to the high G + C percentage group of the so-called *Actinomyces* branch. The LAB are characterized by a fermentative metabolism and gain their energy from the break-down of sugars into metabolites, among which lactic acid is the most important. In the group of homofermentative LAB more than 85% of this acid is produced whereas heterofermentative organisms produce less than 50%.

## LAB in food

In addition to lactic acid, LAB form by-products that are important for the sensory appearance of the food and consist mainly of acetic acid, ethanol, formate and $CO_2$. It is remarkable that each of these products possesses an antimicrobial potential and contributes to a clean fermentation, thereby preventing the growth of pathogenic or putrefactive organisms. Clearly, the formation of acid decreases the pH below those limits that permit the growth of undesirable bacteria. This effect is usually supported by exclusion of $O_2$, which prevents the growth of acid-tolerant yeasts and fungi. The acid formed has further important effects on the food. It contributes to the specific flavour and creates those conditions that allow reactions to proceed that depend on low pH values, e.g. hydrolytic reactions, disproportioning of nitrite, precipitation, coagulation, etc.

It should not be neglected that, when growing in food substrates that should not ferment, LAB may also act as potent spoilage organisms and may cause defects such as sliminess, acidification, gas formation, or off-flavour. These defects per se are not considered to represent health hazards but may even prevent such hazards by indicating mistreatment of a food that might otherwise permit the growth of food pathogens. Thus, the application of "indicative" protective cultures has been recommended, which contain mesophilic LAB and are added to neutral or low-acid food requiring cooling. In case of temperature abuse, the LAB grow, acidify the substrate, indicate the mistreatment and thus protect the consumer against food poisoning [13]. The addition of these cultures does not necessarily result in sensory changes of the treated food exceeding the threshold of perception. As described by Jelle [14], a strain of *Lactobacillus alimentarius* (L-2) without any potential to produce bacteriocins, $H_2O_2$ or diacetyl, suppressed efficiently the growth of spoilage organisms in meat products. Furthermore, the ability of lactobacilli to act as $O_2$ scavengers can be used to prevent lipid oxidation in mayonnaise-based products containing polyunsaturated fatty acids [15].

In fermented foods the establishment of a specific flora and its metabolic performance both depend on factors comprising the following: substrate composition (e.g. carbohydrates, nitrogen sources, vitamins, minerals), water activity, redox potential, pH, temperature and presence of antagonistic compounds or bacteriophages. It can be inferred that the nature of the substrate and the technology applied are the decisive means of influencing the outcome of a specific food fermentation process. In a traditional fermentation process, the indigenous organisms undergo changes after which only a few species or strains predominate and control the process. The successions are remarkably reproducible but may be disturbed by factors such as bacteriophages, presence of inhibitors or absence of the desired fermentation organisms in the contaminating flora. To overcome these uncertainties, the originally uncontrolled process technology was replaced by applying so-called starter cultures. These were originally part of the actively performing fermenting substrate and later became defined cultures containing selected strains with known properties.

The use of starter cultures was the prerequisite for introducing modern industrial technology, which is most advanced in the dairy field and is followed by meat technology and sourdough production, wineries, etc. The starters, furthermore, allowed to study the specific effect of a given organism on the character of the product. It became evident that the production of acid is not the only beneficial metabolic process that contributes to safe and wholesome food. It was observed that the potential of the LAB includes also the formation of specific antagonistic compounds such as $H_2O_2$, fatty acids, benzoeic acid, diacetyl, reuterin and the so-called bacteriocins [16–20]. These compounds may strengthen the primarily important antagonistic effect of substrate acidification or may

even exert specific and unique effects by selectively inhibiting micro-organisms that may cause food spoilage or are food pathogens. The utilization of this type of LAB in culture preparations is the aim of the application of so-called protective cultures that are intended to be employed mainly for non-fermented foods [21–23]. The effect of these cultures may include or potentiate the corresponding effects of the "indicative" protective culture described above.

An additional wholesome effect of LAB arises from their potential for improving the nutritional value of fermented food. Several diverse metabolic properties contribute to the following nutritional effects: increased bioavailability of minerals, production of essential amino acids and vitamins, elimination of endogenous toxins and antinutritional compounds in plant material and "predigestion" of food polymers [24–26]. Their meaning is of different importance in the various foods and will be regarded in this context (vide infra).

A second line of interest in LAB leading to their use in foods is derived from the observation that these organisms are associated with man and animals. Species found in the human gastro-intestinal tract are compiled in Table 2. There is ample evidence that certain species contribute to the well-being of man and animals and, thus, their ingestion as a non-food preparation or part of the diet might be wholesome. Organisms such as *L. acidophilus* and *Bifidobacterium* spp. are examples of species used in food for this purpose. As shown in Table 3, several additional species are applied to make use of their beneficial effects on man. Similar effects are also obtained with micro-organisms outside of the LAB group, and they altogether constitute the so-called probiotics, which were originally defined by Fuller [29] as follows: "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance".

The various fermented foods are characterized by the growth of a very specific population of LAB, the metabolism of which affects the products in a unique way. Below are given some examples of the contributions of LAB to these products.

Table 2. Species of LAB isolated in varying ratios from the human gastro-instestinal tract [5, 27]

| LAB isolated from human gastro-intestinal tract | |
|---|---|
| *Lactobacillus acidophilus* | *Bifidobacterium adolescentis* |
| *L. animalis* | *B. angulatum* |
| *L. brevis* | *B. bifidum* |
| *L. buchneri* | *B. breve* |
| *L. casei* | *B. catenulatum* |
| *L. delbrueckii* | *B. infantis* |
| *L. gasseri* | *B. longum* |
| *L. lactis* | *B. pseudocatenulatum* |
| *L. plantarum* | *B. dentium*[a] |
| *L. reuteri* | *Enterococcus faecalis* |
| *L. ruminis* | *E. faecium* |
| *L. salivarius* | |

[a] Considered as a pathogen

Table 3. LAB used in probiotic products [5, 28]

| LAB in probiotic products | |
|---|---|
| *Lactobacillus acidophilus* | *Streptococcus thermophilus* |
| *L. bulgaricus* | *Bifidobacterium adolescentis* |
| *L. casei* | *B. bifidum* |
| *L. helveticus* | *B. breve* |
| *L. lactis* | *B. infantis* |
| *L. plantarum* | *B. longum* |
| *L. salivarius* | *Enterococcus faecium* |

## Dairy products

When compared with other fields of food fermentation, it is evident that the study of the relationship between milk and LAB is most advanced. This is mainly due to the long tradition of these studies and to the special sensitivity of the substrate to potential microbial health hazards and spoilage. In fact, the first pure culture of a bacterium was obtained by Lister [30] who isolated *Bacterium lactis*, which is now alloted to *Lactococcus lactis*. Furthermore, the pioneering work of Storch, Weigmann and Conn (reviewed by Lundstedt [31]) settled the fundamentals for modern starter technology. Aside from the purpose of ensuring a reproducible fermentation process and to obtain various products at high standard of quality, research is intended to improve the safe and wholesome nature of dairy products by making use of special properties of the LAB.

For two reasons milk is the most suitable substrate for making use of the potential health-promoting effects of LAB. Firstly, there are reports of these effects since the time when Metchnikoff [32] studied the "probiotic effect" of fermented milk. Secondly, the highly developed technology, feasibility of pasteurization and aseptic processing (inoculation, fermentation and preparation of the product) allow work under defined, reproducible conditions. Interference with a fortuitous flora usually does not occur. In addition, it is claimed that LAB in milk association (fermented or not) may exert the following effects [33]: improved lactose utilization; control of intestinal pathogens; antitumour activity and effects on the immune system; control of serum cholesterol level.

### *Improved lactose utilization*

Truly unambigous effects can be observed for improved lactose utilization. Fermented milks or yoghurt are better tolerated by individuals that suffer from lactose maldigestion [33, 34]. This metabolic defect is the normal status of roughly half of the world's adult population and results from a loss of lactase activity in the small intestines during the first 20 years of life. Common symptoms associated with this problem include diarrhoea, flatulence and abdominal pains after consumption of milk.

During the yoghurt fermentation process part of the lactose of the milk is converted into lactic acid by the starter culture usually containing strains of *Lactobacillus*

*delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. In this process the first step, the digestion of the disaccharide lactose by the bacterial enzyme β-galactosidase, is the most important [33]. In addition, the intracellular enzyme β-galactosidase of the micro-organisms apparently is able to pass through the stomach and continues to hydrolyse the lactose in the small intestines. Furthermore, the presence of *L. acidophilus* cells in non-fermented milk significantly improves lactose utilization in test subjects who were lactose maldigestors [35]. Similar results were observed with *B. bifidum* in milk [36]. The mechanism leading to the desired effects in non-fermented milk appears to be similar to that discussed for yoghurt [37].

*Control of intestinal pathogens*

With regard to the further health-promoting claims for LAB in milk association, the experimental evidence is not conclusive for all aspects. This is partly due to the fact that the desired potential differs in the various species or strains of LAB and furthermore the test individuals react differently. The use of orally applied micro-organisms for control of intestinal pathogens is a major aim of the concept of probiotics, and their use receives growing interest. Although various interactions between probiotic LAB and the microbial flora of the host were described, most of the wholesome effects are not fully understood and are still under investigations. The present status is characterized by Renner [38] as follows: "We are still far away from a point where all these claimed beneficial effects could be considered to be well established from a scientific view". As pointed out by Morelli [39], this characterization is not to be considered discouraging or negative towards the use of probiotics: "It simply will to stress the enormous potential of providing living microorganisms with healthy effects on humans and animals. Molecular biology may provide the means by which a number of the classical problems of research in probiotics can be solved".

The desired effect of probiotics results from the observation that the dominant autochthonous micro-organisms in man and animals can regulate the indigenous flora and can interfere with pathogenic bacteria which cross the gut as dicussed by Fuller [29]. This concept has led to the inoculation of selected microbial strains to promote the resistance to infectious disease as cited by Tannock [36]. The following mechanisms have been proposed to be responsible for the exclusion from the intestines of non-indigenous organisms [40]:

1. Competition for nutrients present in limited quantities.
2. Competition for intestinal mucosal association sites.
3. Creation of selective environmental conditions.
4. Formation of antagonistic compounds.

To be effective, a probiotic should share some of these properties of the autochthonous flora, and it is discussed that further wholesome effects may be attributed to either special enzyme activities or stimulation of the immune system.

Amongst the probiotic LAB, strains were identified producing antagonistic activities against other micro-organisms, which are now defined as bacteriocins [20], i.e. proteinaceous compounds with a bactericidal mode of action and a narrow inhibitory spectrum. For LAB this spectrum may be relatively large and cover species outside the genus of the bacteriocin producer. "Active" products such as lactacin F [41] and acidophilin [42] are formed by *L. acidophilus* strains, but the effect of such bacteriocins on the probiotic interactions of some LAB still remains to be elucidated.

Dairy products fermented by lactobacilli were suggested to control diarrhoea. *L. acidophilus* and *B. bifidum* have received most attention as experimental data are consistent with an inhibitory effect on commonly known food-borne pathogens (reviewed by Gilliland [33]). Nader de Macias et al. [43] observed a high resistance to *Escherichia coli* and *Listeria monocytogenes* infections by feeding mice with milk fermented with *Lactobacillus acidophilus* or *L. casei*, which were isolated from a human source and therefore able to survive and become established in the human gastro-intestinal tract. Results indicated an immunostimulant effect produced by the fermented milk [44]. Further studies demonstrated similar protective effects against infections caused by *Salmonella* [45] or *Shigella* [46].

It appears likely that a micro-organism with a potentially useful specific property related to health promotion of man or animals is unable to maintain itself in the host. The micro-organism in question may lack the genetic potential for restistance against the environmental conditions and/or for adherence and colonisation. If genes are known to be responsible for colonising factors, such as attachment, and can be transferred to the probiotic strain, an ideal combination is engineered [47]. An example for such a genetic manipulation is described by McCarthy et al. [48]. The potential of molecular genetics to study micro-organisms in relation to intestinal ecology has been reviewed by Tannock [49].

*Antitumour activity and effects on the immune system*

Anticarcinogenic or antimutagenic activity has been attributed to various LAB [50–53]. Some of these activities are apparently due to compounds or substances produced during growth of probiotic micro-organisms. The following mechanisms are discussed to explain the anticarcinogenic properties of lactobacilli:

1. A direct conversion or elimination of procarcinogenic or carcinogenic compounds (e.g. nitrite).
2. A reduction of the level of the faecal enzymes β-glucuronidase, azoreductase and nitroreductase responsible for the conversion of procarcinogens into carcinogens.
3. Stimulation of the immune system of the host.

Consumption of milk containing cells of *L. acidophilus* results in a significant reduction of the activity of all three procarcinogenic faecal enzymes mentioned above [53]. These observations may suggest an indirect effect, since the three enzymes are mainly of bacterial origin. This beneficial effect could be explained by a favourable

change in the composition of the intestinal flora as a result of the introduction of the lactobacilli [26].

An activation of the immune system is considered not only to contribute to the control of intestinal pathogens treated above, but also to antitumor effects. As reviewed by Huis in't Veld et al. [24], a recent case control in France and in the Netherlands observed a protective effect of yoghurt, buttermilk and curds on the risk of breast cancer. It is discussed that the ingested micro-organisms or their antigens penetrate the epithelial barrier of the intestines and stimulate the immuno-competent cells associated with Peyer's patches, the mesenteric lymph nodes and the spleen [24]. LAB in fermented dairy products or of other origin trigger the local and systemic immune system. Perdigon et al. [44] demonstrated the activation of the immune system in mice after feeding *L. casei*, and showed that the activity of macrophages increased rapidly by the effect of living cells. De Simone et al. [54] found *L. acidophilus, L. delbrueckii* subsp. *bulgaricus* and bifidobacteria to influence the regulation of gamma-interferon production by human peripheral blood lymphocytes in vitro. This interferon exhibits antiviral and antiproliferative effects and can activate killer cells.

*Control of serum cholesterol levels*

As hypercholesteremia is an important risk factor for development of cardiovascular disease in man, reports describing the effects of fermented foods on the reduction of the serum cholesterol concentration attract much attention. This type of beneficial influence was also reported for the fermented dairy products or cells of *L. acidophilus* (reviewed by Gilliland [33]; [55]). The results are, however, rather inconsistent and unambiguous data are still lacking.

Various lactobacilli present in the intestinal tract are able to deconjugate the bile acids taurocholate and glycocholate. This reaction releases cholesterol from the micelle association rendering it unavailable for absorption. Therefore, the introduction of lactobacilli endowed with this activity could reduce the cholesterol uptake from the intestines [26]. Furthermore, production of new bile acids to replace the excreted, deconjugated bile acids may also reduce the level of the precursor cholesterol. However, it appears doubtful whether the presence in the small bowel of LAB with deconjugating activity is a desirable property. According to Marteau and Rambaud [56] such a type of LAB should cause malabsorption and diarrhoea. Certain strains of *L. acidophilus* are reported to assimilate cholesterol in the intestines. It remains to be shown to what extent this effect can be verified in man by employing these strains or others isolated from the indigenous human flora.

## Meat products

Fermentation of meat is a traditional method for obtaining keepable products from the substrate meat, which otherwise is extremely sensitive to spoilage. Fermented sausages are the main products and LAB either alone or in combination with micrococci, yeasts or moulds are essential for the fermentation process [57]. The raw materials cannot be pasteurized and, therefore, the fermentation flora has to compete with the fortuitous organisms. LAB decrease the pH in the sausages, whereby major effects are exerted on texture, colour, flavour and, above all on food safety [57]. This latter effect is supported by low water activity and presence of nitrite. The application of starter cultures has become a means of controlling the fermentation process. The fermented products have a safe reputation but, nevertheless, the process technology has to be directed to the prevention of the growth or even the elimination of food pathogens such as *Staphylococcus aureus, Listeria monocytogenes* and *Salmonella*.

Since bacteriocins from meat-associated lactobacilli or pediococci have usually a strong antagonistic effect on *L. monocytogenes*, it is suggested to apply these LAB in starter cultures. In fact, studies with bacteriocin-forming pediococci and lactobacilli indicate that this antilisteria effect can be observed in meat products [58, 59]. For example, the number of *L. monocytogenes* decreased significantly when a bacteriocin-producing strain of *Lactobacillus sake* was applied to spreadable sausages. It is however, important that starter strains with the potential for bacteriocin production do not interfere with the safe performance of micrococci as desired components of the starter preparation. On the other hand, the bacteriocin-producing strains are extremely competitive during sausage fermentation [60] and lead to a "clean" fermentation without any major growth of the fortuitous LAB flora. This observation may be considered an indication of the efficiency of bacteriocins during sausage fermentation.

As novel approaches for making use of the beneficial properties of LAB, protective cultures have been applied to meat and meat products. As reviewed by Geisen et al. [61], the growth of undesired bacteria can be reduced when these cultures were applied to the surfaces of products like non-fermented sausages, carcasses or in minced meat.

## Fermented food of plant origin

Different from fermented food of animal origin, the majority of those derived from plants are low in protein content. In the case of fermented cereal products, their special dietary value rests primarily on the carbohydrate content, whereas vitamins, minerals and dietary fibre characterize food derived from vegetables. It should not be neglected that in Asian countries high-protein fermented food from legumes are known [62].

The nutritional value of food plants depends not only on the amounts of nutrients present but also on the presence of antinutritional factors that interfere with the availability or utilization of the nutrients. Well-known examples that are related to the beneficial activity of LAB are: the metal-chelating compounds phytate and oxalate in many plants; goitrins in the Brassicaceae, which decrease the iodine uptake; and trypsin inhibitors in cereals and legumes, which decrease the digestibility of protein.

Well-being may further be affected by flatulence resulting from high contents of raffinose or stachyose, which require α-galactosidase for their digestion. This activity is absent in man but present in some LAB. In addition, plants may contain potent toxins that cause intoxication when raw plants are eaten. During fermentation, compounds such as cyanogens, glucosinolates or heme agglutenins have been found to become inactivated.

The poor availability of iron in plant food is of special importance as anaemia is a common health problem in developing countries, with young children and women being most vulnerable [63]. Results of a number of studies have suggested that iron deficiency is the major cause of the anaemia, despite the presence of abundant amounts of iron in foods. Most of the iron ingested is derived from plant sources and generally this non-heme iron is not readily available. The low bioavailability of iron from vegetables and grains is partly explained by the presence of phytate (inositol hexaphosphate). Svanberg et al. [63] observed an increase in soluble iron in fermented vegetables corresponding to a complete reduction in the amount of inositol-hexaphosphate in fresh vegetables during lactic acid fermentation. Furthermore, the amount of soluble iron is increased in phytate-rich meals when fermented vegetables were added. This indicated the formation of iron-promoting factor(s) in lactic-acid-fermented vegetables.

Recently, Kohler and Holzapfel [64] have shown that LAB possess phytase activity. It appears likely that this activity is enhanced by the decrease in pH during vegetable fermentation, which activates the endogenous plant phytase. To some degree lactate per se and amino acids such as lysine may chelate iron and thus, contribute to an improved iron availability in fermented plant foods [25]. An absence in LAB of iron-providing mechanisms is consistent with the observation that iron is not essential for LAB. This property is considered to count as a factor contributing to the special competitiveness of LAB in mixed populations where iron supply is limited [65].

Lactic acid fermentation usually preserves vitamins present in the raw material very well. This is mainly due to the anaerobic conditions and the low pH values. Most LAB require vitamins for growth but some strains increase the B-vitamin content of fermented products. For example, the nutritive value of cornmeal fermented by endogenous LAB increased as a result of the increase in the vitamins riboflavin, niacin and, furthermore, in the essential amino acids lysine, tryptophane and methionine. This effect is dependent on the fermentation temperatures [66]. The best value for formation of the three amino acids was determined at fermentation at 45° C. This type of investigation has shown that there is a potential for using lysine-excreting mutants of LAB to improve the nutritive value of foods, such as cereal products, which usually have a low content of this essential amino acid [67]. Mutants of *L. acidophilus, L. bulgaricus* and *L. plantarum* have been suggested for such use.

At present, the fermentation of plant materials is still an uncontrolled process, meaning that starter cultures are not applied at a significant degree. An exception is the production of sour dough for which process several preparations are available [68]. Up to now organisms potentially used in starter cultures for the fermentation of plant materials have been selected for technological and sensorial reasons. On the other hand, to make use of the potential of selected LAB to improve the nutritional value of the food has not yet attained any practical importance. This potential may also include genetically modified starter cultures.

## Future aspects

The increased interest in research of LAB has broadened our knowledge of their limitations and potentials. In addition to the traditional fields of application, new areas have been opened for their use. For example, the US National Academy of Sciences report "Designing Foods" [69] discussed future trends and developments in the creation of nutritionally based products for the general population. Among other items of interest the report included the development of genetically engineered dairy starters that will digest the cholesterol of butterfat during fermentation of cheese, resulting in a no(low)-cholesterol product; and the use of micro-organisms to remove specific amino acids from protein, to benefit those inborn errors of metabolism [70].

The application of probiotics, protective cultures and selection of strains with specific nutritional properties useful in the fermentation process have already been mentioned. In particular, the study of bacteriocins from LAB has been intensified and has yielded during the last 15 years a considerable number of well characterized compounds produced by species belonging to virtually all genera of LAB (reviewed by Klaenhammer [20]; Nettles and Barefoot [71]; Sudirman et al. [72]). In Table 4 those bacteriocins are grouped according to their chemical

Table 4. Grouping of structurally characterized bacteriocins of LAB

| Chemical structure | Bacteriocins of LAB | References |
|---|---|---|
| Small, unmodified peptides | Pediocin PA-1.0 | [73–75] |
| | Sakacin P | [76–77] |
| | Curvacin A/Sakacin A | [76, 78]/[79] |
| | Bavaricin A | [80] |
| | Lactacin F | [81, 82] |
| | Lactococcin A | [83–85] |
| | Lactococcin B | [86] |
| | Leucocin A-UAL 187 | [87] |
| | Mesentericin Y105 | [88] |
| Lantibiotics | Nisin | [71] |
| | Lactocin S | [89, 90] |
| | Carnocin U149 | [91] |
| 2-peptide-bacteriocins | Lactococcin G | [92] |
| Protein-bacteriocins | Helveticin J | [93] |
| Glycoproteins | Leuconocin S* | [94] |
| | Plantaricin S* | [95] |

* Structurally not yet defined

structure. It is suggested that the bacteriocin producing strains may serve for the following practical purposes:

1. Development of protective cultures with the potential to inhibit spoilage organisms and food pathogens.
2. Development of probiotics.
3. Application of the purified bacteriocins as natural preservatives.
4. Development of starter cultures with special competitiveness in a fermentation process.

Except for nisin, which has been in use for preservative purposes since over 40 years [96], no other bacteriocin or producer organism has found deliberate practical application. As adequate methods for genetic modification of LAB have been developed, it is possible to further improve their health promoting potential. With regard to make use of bacteriocin producing strains, the following genetic modifications may be performed [97]:

1. Cloning the genes for production and immunity onto a high copy-plasmid resulting in an increase of the gene product.
2. Optimized production by factors affecting transcription, such as promoter strength and positive and negative regulators.
3. Genetically altering the structure of bacteriocins to broaden their activity spectra.

It has been proposed to construct probiotics from strains with strong ability to adhere to the intestines and to endow them with genes coding, for example, for bacteriocin production or beneficial enzyme activities. In addition, the construction is possible of strains that are best adapted to a given food environment by transfer and combination of useful genes into starter organism. This modification may also include properties of primarily technological importance such as phage resistance. The safe performance of a phage-resistant strain prevents the risk of malfermentation and thereafter the growth of food pathogens. The prevention of this latter risk was also the aim of the transfer of the lysostaphin gene to *L. casei* [98], which activity inhibits the growth of *Staphylococcus aureus*. Furthermore, the lysis gene from a phage of *Listeria monocytogenes* was isolated and transferred to *Lactococcus lactis*. The new strain was able to lyse *Listeria monocytogenes* and thus can be beneficially used in starter cultures (M. Gasson, personal communication). The application of genetically modified starter organisms depends on their acceptance by the consumer. Up to now the potential of LAB has not yet been included in public discussion and may provide an example of genetic modification in food field with beneficial effects on the wholesomeness of food.

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